Tests for Synergism Between Nicotine and Phthalonitrile and Between Nicotine and 2,3,4,5,6-Pentachloroanisole

In our search for synergists for nicotine, we subject a mixture of nicotine and the adjunct to a screening test, following methods already described (Swingle 1943, Mayer *et al.*, 1945, 1946). Any compound which stays on the screen is presumed to have possible synergism, and is then included in tests designed to produce data for dosage-mortality curves. The curves are analyzed by the short method of Wadley (1945). Of 200 chemicals examined to date, a number showed possible synergism (Mayer *et al.*, 1949, Table 1). This paper reports the dosage-mortality data for two of them, phthalonitrile and 2,3,4,5,6-pentachloroanisole, and the calculations for synergism.

Phthalonitrile is itself an insecticide (Schecter & Haller, 1940; Swingle 1941, 1944), as is also pentachloroanisole (Carswell 1944). These facts are confirmed in the present study, wherein there was occasion to measure the toxicity of the compounds alone.

Insect and Foliage Tests.—Nicotine sulfate, nicotine bentonite, and cuprous nicotine cyanide (Mayer *et al.*, 1945) were used at concentrations of nicotine ranging from 0.02 to 10 per cent in attapulgite as a dust carrier, the dosages ranging from 0.2 to 10.0 micrograms per square centimeter.

The insects, their stages, and the foliage used were: Third instars of diamondback moth, *Plutella maculipennis* (Curt.), crucifers; fourth instars of the armyworm, *Cirphis unipuncta* (Haw.), corn and barley; third instars of the celery leaf tier, *Phlyctaenia rubigalis* (Guen.), celery and Swiss chard; fourth instars of the California oakworm, *Phryganidia californica* (Pack.), live oak; third instars of the green dock beetle, *Gastrophysa cyanea* (Melsh.), dock; day-old nymphs of the pea aphid, *Macrosiphum pisi* (Kltb.), Windsor bean; adults of a pomace fly, *Drosophila sp.*); and adults of the greenhouse thrips, *Heliothrips haemorrhoidalis* (Bouche), citrus leaves. Larvae of the California oakworm and eggs and larvae of the green dock beetle were collected in the field. All other insects were reared in the laboratory.

The materials were tested against the lepidopterous and beetle larvae by infesting dusted foliage in 9-cm. petri dishes. All leaves were dipped in a 0.5 per cent saponin solution before dust was applied so that none of the particles would drop off in manipulation.

Uniform ages of pea aphid nymphs were obtained by infesting a Windsor bean plant with apterous adults and allowing them to reproduce overnight, after which the adults were removed. The following day the nymphs, which were always located on the under sides of the leaves, were treated by inverting the plant and allowing the dust to settle directly on them.

Greenhouse thrips were tested by confining them in inverted shell vials held to a dusted citrus leaf on a board by rubber bands. The stem of the leaf was immersed in a vial of water.

Tests against pomace fly adults were also carried out in shell vials. Filter papers were cut to line the inner walls of the vials, dipped in ethyl alcohol, and dusted while they were still wet. The papers were then placed in the vials, after which a few grains of granulated sugar and the insects were added. Cheesecloth caps were used, and the vials were kept in a desiccator containing a small amount of water.

The armyworm fumigation test was made by placing one-eighth teaspoon of the material between two sheets of filter paper pressed into the lid of a petri dish containing the insects and untreated foliage. The chemicals, if volatile, soon filled the dishes with their vapors.

Final mortality counts were made at the end of 2 days for the pea aphid and at the end of 3 days for all others.

Foliage injury tests were made by spraying mixtures of the materials on five kinds of plants—corn, cucumber, potato, cabbage, and wax bean. Concentrations were (1) 0.1 per cent of nicotine sulfate

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[3] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

Table 1.—Complete insecticidal data and calculations for group 7 in Table 2 and Figure 1,A.

Adjunct: Phthalonitrile
Insect: Diamondback moth, L-3

Compound of nicotine: Sulfate
Carrier: Attapulgite. Leaves wet with 0.5% saponin before dusting

Mortality Data for Toxicants Singly

Date of Set	Number of Insects	Nicotine	Mortality		Number of Insects	Phthalonitrile	Mortality	
		μg./cm²	Per Cent	Probits		μg./cm²	Per Cent	Probits
10/28/46	18	3.7	78	5.77	27	4.2	52	5.05
	26	7.6	96	6.75	29	5.7	76	5.71
					28	7.6	82	5.92
11/ 4/46	18	3.4	61	5.28	20	4.2	45	4.87
	20	5.8	90	6.28	23	5.6	56	5.15
	22	7.7	91	6.34	21	7.6	86	6.08

Mortality Data for Toxicants Combined. Wadley Calculation
Nicotine equivalence = 0.68 at LD50[1]

Date of Set	Number of Insects	Mixture		Nicotine Equivalent[2]	Mortality	
		Nicotine	PHTH			
		μg./cm²	μg./cm²	μg./cm²	Per Cent	Probits
10/28/46	23	1.08	0.54	1.44	26	4.36
	24	1.44	.72	1.91	42	4.80
	26	2.10	1.05	2.79	81	5.88
11/ 4/46	23	.98	.49	1.30	17	4.05
	22	1.56	.78	2.07	50	5.00
	18	2.04	1.02	2.71	72	5.58

$$\text{Log ratio} = \frac{\log 2.9 - \log 2.02}{0.06} = 2.6$$

[1] Nicotine equivalence at LD50 = $\frac{2.9 \text{ (dosage from curve for nicotine alone at probit 5)}}{4.3 \text{ (dosage from curve for adjunct alone at probit 5)}} = 0.68$.

[2] Nicotine equivalent = nicotine dosage + (0.68 × adjunct dosage).

plus 0.3 per cent of phthalonitrile and (2) 0.1 per cent of nicotine sulfate plus 0.15 per cent of pentachloroanisole. They were sprayed on the first and seventh days of the test. No injury resulted in 14 days.

Statistical Methods.—To determine synergism by the Wadley method, it is necessary to establish three mortality curves—for nicotine alone, for the adjunct alone, and for the mixture. Each curve was comprised of three to five dosages; about 30 insects were used with each dosage. The term "set" encompasses the three curves established on a given day. In many cases a single set was plotted (Fig. 1, B). When, however, the data for several sets were consistent when plotted, they were used together (Fig. 1, A, C, and D). Plotting can be done on log-probability paper (Wadley 1945), but it is preferable to convert per cent mortality to probits (Bliss 1935) and plot on semi-log paper.

Mere inspection of the charts gives a rough idea of the presence or absence of synergism. The prime consideration, of course, is the displacement of the nicotine equivalent curve to the left of the nicotine curve. An arbitrary system is used to indicate the degree of synergism as determined by inspection. If the two curves coincide, or if the nicotine equivalent curve is to the right, a minus sign is given; if the nicotine equivalent curve is slightly to the left, one plus sign; if definitely to the left, and especially if the curve is steeper than that for nicotine alone, two or three plus signs. Weight is also given to the number of sets of data, their scatter, and their position on the mortality axis. In this system, one plus is not significant; two indicate significant synergism; and three, marked synergism. If the nicotine equivalent curve is to the

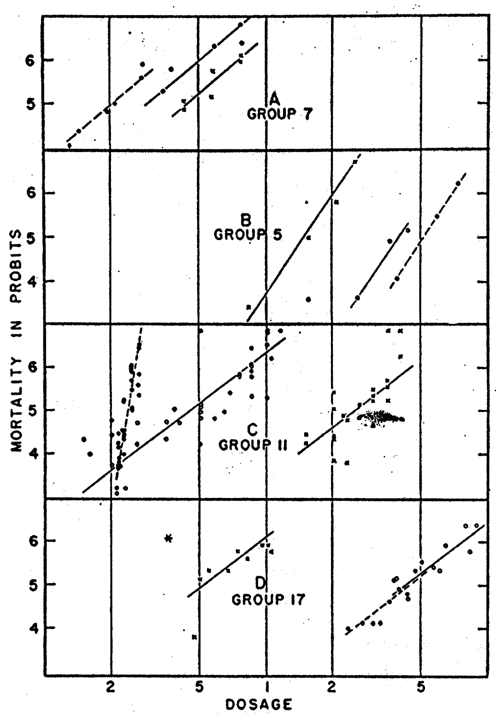

Fig. 1.—Dosage-mortality curves which show the four patterns of synergism of phthalonitrile and pentachloroanisole for nicotine. A, B, and C, phthalonitrile tested on the diamondback moth, greenhouse thrips, and the armyworm, respectively; D, pentachloroanisole tested on the diamondback moth. Open circles indicate nicotine alone, crosses indicate adjunct alone, and broken lines indicate the nicotine equivalent of the mixture. Dosages of toxicants are in micrograms per square centimeter, except in the section of D marked with an asterisk, where they are one-tenth those shown on the abscissa.

right, the same degrees of antagonisms are indicated.

Because of the voluminous data that result from tests of this kind, the data and calculations for only one representative group are presented here (Table 1). Table 2 is a summary of the 18 groups of data.

Calculation of the Mathematical Value for Synergism.—Wadley (1945) states that the interpretation of synergism should be in terms of the standard errors of the observations, but he gives no specific way in which a mathematical value for the degree of synergism can be attained. Finney (1947) proposes such a method, but since his formulas encompass all known factors the calculations are too long and involved for practical application to a long series of data.

Dr. Wadley became interested in our data, and kindly made the Finney calculations not only for four groups of data in table 2 (groups 5, 7, 9 and 15) but for five others obtained under similar test conditions but not included in this paper. He then proposed (1949) the log ratio as a simple short-cut calculation. From the eye-fitted regression lines, the dosages at probit 5 (50 per cent mortality) for nicotine (M_A) and for nicotine equivalent (M_B) are read and converted to logs. Then

$$\log\ \text{ratio} = \frac{\log\ M_B - \log\ M_A}{0.06}$$

The 0.06 is a generalized error in logs deduced from the nine sets of data. Dr. Wadley is careful to point out that this generalized error holds only for the test conditions used here; it would be modified by use of more insects, more replication, radically different slopes, and other factors.[1]

The calculation of log ratio is given at the bottom of table 1. Table 2 gives the degree of synergism for all groups of data, either calculated by log ratio or determined by inspection when the data were not calculable.

Table 3 gives the results for the nine sets of data by the two methods—the standard Finney "T" and the log ratio. In general the agreement is good. The average value by the Finney method is 3.64, and by log ratio it is 3.44. The agreement of the short-cut method with the standard is satisfactory for the purposes in hand—the handling of a large amount of data to determine whether any apparent synergism is significant, and to obtain a single value for synergism.

In the short-cut calculation, the preliminary procedure is the same as that described by Wadley except for the use of probits. The chart is inspected for synergism with the following guiding principles.

a. If the nicotine and the nicotine equivalent curves obviously overlap, synergism is absent.
b. If the two curves are markedly divergent, synergism (or antagonism) is present, but its extent cannot be calculated by the log-ratio method.
c. If the two curves are separated and are more or less parallel, or can be made so by reasonable adjustment, the degree of significance of the separation can be calculated either by the Finney formulas or by the log ratio formula.

[1] Since the preparation of this manuscript, Wadley has published his procedure. See literature cited, Wadley (1949).

Table 2.—Summary of the evaluation of two nicotine synergists.

Group	Insect	Application[1]	Compound of Nicotine[2]	Sets	Insects	Synergism Pattern (Fig. 1)	Degree of Synergism[3]
				Number	*Number*		
			Phthalonitrile				
1	Pea aphid, 24 hrs. old	D	S	5	1780	C	++
2	Pea aphid, 24 hrs. old	D	B	1	130	C	++
3	Pomace fly, instar A	P	S	4	896	D	−
4	Greenhouse thrips, instar A	D	S	4	1520	D	−
5	Greenhouse thrips, instar A	D	B	1	1329	B	−1.9
6	Diamondback moth, L-3	D	S	1	278	C	+
7	Diamondback moth, L-3	D	S	2	388	A	2.6
8	Diamondback moth, L-3	D	Cu-Cy	6	753	A	1.5
9	Diamondback moth, L-3	D	B	1	210	A	3.4
10	Armyworm, L-4	D	S	8	854	C	++
11	Armyworm, L-4	D	S	9	2817	C	+++
12	Armyworm, L-4	D	S	1	265	C	+++
13	Armyworm, L-4	F	S	1	208	C	++
14	Green dock beetle, L-3	D	S	4	892	C	+
15	California oakworm, L-4	D	S	1	261	A	3.2
16	Celery leaf tier, L-3	D	S	1	212	A	+
			2,3,4,5,6-Pentachloroanisole				
17	Diamondback moth. L-3	D	S	3	661	D	−
18	Armyworm, L-4	D	S	9	3227	D	−

[1] D=dusted leaves; P=vials lined with filter paper impregnated with toxicant; F=fumigating action from isolated toxicant in petri dish.
[2] S=nicotine sulfate; B=nicotine alkaloid on bentonite; Cu-Cy=cuprous nicotine cyanide (Mayer *et al.*, 1945).
[3] See text for explanation.

There are, of course, shortcomings in the short cut. It generalizes all sources of errors, such as the number of insects used at each dosage, the weighting of points in respect of their distance from 50 per cent mortality, the slopes of the regression lines, and natural mortalities, although the latter may easily be included. The log ratio method assumes that these factors are constant. If they change appreciably, the generalized error would have to be recalculated.

The value of the "T" or of the log ratio that is to be deemed significant is open to judgement. A value of 2 is probably significant at the 5 per cent level and 2.6 at 1 per cent; 3 is highly conservative.

The curves for our present data fall into the four synergism patterns shown in figure 1. A is positive, calculable; B is negative, calculable; C is positive, significant, not calculable; D indicates no synergism. Table 2 gives the pattern for each group of data.

Conclusions.—We conclude that, under the test conditions used, mixtures of phthalonitrile and nicotine sulfate or bentonite showed definite synergism against the pea aphid (groups 1 and 2, table 2), armyworm (10, 11, 12, and 13), California oakworm (15), and diamondback moth (7 and 9); doubtful or no synergism against the green dock beetle (14), celery leaf tier (16), and pomace fly (3); and antagonism against the greenhouse thrips (5). Antagonism may also be construed in the lower portions of those nicotine equivalent curves which cross the nicotine curves (groups 1, 6, and 14 (not shown in figure 1)). It should

Table 3.—Comparison of calculations for synergism by Finney's method and by log ratio. Calculations made by F. M. Wadley.

Data	Finney "T"	Log Ratio
Group 5, greenhouse thrips	−2+	+1.9
Group 7, diamondback moth	3.0	2.6
Group 9, diamondback moth	3.2	3.4
Group 15, California oakworm	3.4	3.2
Bis-(p-chlorophenyl) sulfide, armyworm	4.8	3.1
Bis-(p-chlorophenyl) sulphide diamondback moth	3.3	4.6
Bis-(p-chlorophenyl) sulphide California oakworm	5.6	6.3
Tridecanenitrile, armyworm	−5.2	−2.5
2-Cyclohexylcyclohexylamine, armyworm	−2+	−3.1

be pointed out, however, that in these curves the lower portion involves a higher nicotine-phthalonitrile ratio, and the upper portion a lower ratio. Conceivably there is an optimal range of ratios for effective synergism. Bliss (1939) considers this ratio an important factor in synergism.

Mixtures of nicotine sulfate and pentachloroanisole showed neither synergism nor antagonism against the diamondback moth and the armyworm.

Perhaps the most conspicuous thing in the data is the greatly increased slope of the nicotine equivalent curve in the 8 groups showing pattern C. This might indicate a chemical combination between and phthalonitrile, more probably inside the insect than outside, resulting in a more toxic compound, a phenomenon which Horsfall (1945) called potentiated synergism.

We conclude, in general, that phthalonitrile and nicotine are synergistic against insects (McGovran *et al.*, 1948) but that insect specificity again is in the picture.

SUMMARY.—Under the test conditions used, mixtures of nicotine and phthalonitrile were definitely synergistic against the pea aphid, armyworm, California oakworm, and diamondback moth. They showed doubtful or no synergism against the green dock beetle, celery leaf tier, and pomace fly. They showed antagonism against greenhouse thrips.

Mixtures of nicotine and pentachloroanisole showed neither synergism nor antagonism against the diamondback moth and the armyworm.

In addition to the Finney method for calculating synergism data, a shortcut procedure devised by F. M. Wadley is presented. Each was applied to four groups of present data and to five other groups not included in this paper. The numerical values obtained confirm the conclusions of synergism and antagonism in these cases.

Sprays of the mixtures on five species of plants caused no injury to the foliage.

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